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Co-suppression of *NbClpC1* and *NbClpC2* in *Nicotiana benthamiana* lowers photosynthetic capacity via altered leaf structure

Md. Sarafat Ali[1,†], Youngjae Yu[2,†], Wook Oh[3], Ji Yoon Cho[3], Jinhee Choi[1], Radhika Dhakal[1], Youn-Il Park[2], Kwang-Hyun Baek[1*]

[1]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Republic of Korea
[2]Department of Biological Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea
[3]Department of Horticulture & Life Science, Yeungnam University, Gyeongsan 712-749, Republic of Korea

†Both authors contributed equally to this work
***Corresponding author: khbaek@ynu.ac.kr**

Abstract

The Clp protease composed of more than 15 proteins, is the central protein degradation machinery in the plastids of a plant cell which plays a similar role as proteasomes in cytoplasm and nucleus. ClpC1 and ClpC2 are two chaperonic proteins for the Clp protease and share more than 90% similarities in the nucleotide and amino acid sequences. In this study, we investigated the functions of ClpC1 and ClpC2 in photosynthesis by co-suppression of both genes (*NbClpC1* and *NbClpC2*, *NbClpC1*/*C2)* in *Nicotiana benthamiana* using virus-induced gene silencing (VIGS) technique. Co-suppression of *NbClpC1*/*C2* in *N. benthamiana* resulted in aberrant structure with severe chlorotic leaves, stunted growth and reduced numbers of stomata and in lowered photosynthetic capacity. Leaf CO_2 assimilation rate, the maximum quantum yield of photosystem II (Fv/Fm), chlorophyll and carotenoid contents were also significantly lower in the leaves of *NbClpC1*/*C2* co-suppressed *N. benthamiana* than those in the control. Microscopic analysis revealed that *NbClpC1*/*C2* co-suppressed leaves had coarsely packed mesophyll cells. Our findings strongly suggest that ClpC1 and ClpC2 play a pivotal role in photosynthetic competence by affecting the proper structure of leaves and numbers of stomata.

Keywords: Clp protease, *ClpC1*, *ClpC2*, Photosynthesis, Virus-induced gene silencing, *Nicotiana benthamiana*.
Abbreviations: CRSP_CO_2 RESPONSE SECRETED PROTEASE, EPF_EPIDERMAL PATTERING FACTOR, GFP_green fluorescent protein, *NbClpC1*/*C2_NbClpC1 and NbClpC2*, VIGS_virus-induced gene silencing.

Introduction

As sessile organisms, plants are persistently exposed to stress-induced factors, which can cause protein abnormalities. Abnormal proteins also arise in cells as a result of mutations, errors in protein synthesis or folding, spontaneous denaturation, diseases or oxidative damage. If not removed, these damaged proteins eventually poison the cell, often by forming large insoluble aggregates. The abnormal proteins within a cell are usually controlled by two mechanisms, molecular chaperones and proteases (Sjogren et al., 2014). Chaperones are essential to plant growth and development as they help the cell recover from stresses by either repairing damaged proteins (protein refolding) or helping to degrade them, restoring protein homeostasis and promoting cell survival (Katiyar-Agarwal et al., 2003). Many chaperones can work independently, while others require interaction with proteolytic complexes (Doyle et al., 2013). In addition, proteases such as proteasome in cytoplasm and Clp protease in chloroplasts can cleave abnormal proteins and facilitate the recycling of valuable amino acids. Plastids have a dynamic proteolytic system in which proteases play an important role in the process of protein precursors, degradation of incomplete proteins lacking cofactors, and degradation and reactivation of damaged proteins. There are three major types of plastid proteases, ATP-dependent FtsH protease (Adam et al., 2006; Kidric et al., 2014; Liu et al., 2010) and Clp protease (Adam et al., 2006; Kidric et al., 2014;) and ATP-independent Deg/HtrA protease (Huesgen et al., 2009; Kidric et al., 2014; Sun et al., 2007; Sun et al., 2010). FtsH protease is located in the thylakoid membrane (Kato and Sakamoto, 2010; Kidric et al., 2014; Sakamoto, 2006), while Deg/HtrA protease is present in both lumenal (Deg1, 5, 8) and stromal membrane (Deg2, 7) (Huesgen et al., 2009; Kapri-Pardes et al., 2007; Sun et al., 2007; Sun et al., 2010) and Clp protease is localized in the stroma with some association to the inner membrane of the chloroplast (Peltier et al., 2004; Sjogren et al., 2014; Zheng et al., 2002). The Clp protease is a unique proteolytic system similar to the proteasome in the cytoplasm and nucleus. These proteases are composed of two major components, a barrel-shaped tetradecameric protease core with catalytic sites sequestered inside the complex and hexameric ring-like ATP-dependent chaperones (Baker and Sauer, 2006; Striebel et al., 2009). Protein substrates are unfolded by the hexameric chaperone ring and pushed into the chamber of the stacked heptameric rings of the Clp core. Cleaved peptides exit through axial channels or via polar lateral openings (Peltier et al., 2004). The Clp protease in higher plants represented by *Arabidopsis thaliana* is composed of more than 15 proteins in the plastid, but little is

known about their precise functions. The 15 proteins include three Clp AAA+ chaperones (ClpC1, ClpC2 and ClpD), five serine type Clp proteolytic subunits (ClpP1, ClpP3, ClpP4, ClpP5 and ClpP6), two proteins as a core (ClpS1 and ClpS2), four non-proteolytic regulatory subunits (ClpR1, ClpR2, ClpR3 and ClpR4) and two other proteins (ClpT1 and ClpT2) (Adam et al., 2006; Peltier et al., 2004; Sjogren et al., 2014). With the exception of the *ClpP1* gene in the plastidial genome, other Clp protease subunits are encoded by the nuclear genome. As chaperonic proteins, ClpC1 and ClpC2 play important roles in protein quality control (Hengge and Bukau, 2003; Sjogren et al., 2014; Trosch et al., 2015). There is evidence that overexpression of *ClpC2* in *Arabidopsis* is able to complement the *clpc1* mutant to the wild type (Kovacheva et al., 2007). Mutant formed by *ClpC2* knockout via T-DNA insertion did not show distinct phenotypic changes (Kovacheva et al., 2007; Park and Rodermel, 2004). ClpC1 seems to be more important than ClpC2 in their biological functions, as shown by *clpc1* mutant having clearly stunted growth with chlorosis and a substantial reduction in the accumulation of photosystem I (PSI) and photosystem II (PSII) complexes (Trosch et al., 2015).

ClpC1 and ClpC2 perform many different functions in the chloroplasts of a plant cell. In cyanobacteria, ClpC1 and ClpC2 play significant roles in cell viability and phototrophic growth (Clarke and Eriksson, 1996). Simultaneous repression of the expression of *ClpC1* and *ClpC2* genes using the antisense technique in tobacco failed to produce viable cell lines (Shanklin et al., 1995). ClpC1 and ClpC2 generally play a housekeeping role within the chloroplasts by acting as either independent chaperones or within a Clp protease (Trosch et al., 2015). Additionally, they are important factors in the import of nuclear encoded cytosolic protein precursors into the chloroplast and degradation of aberrant polypeptides with recycling of valuable amino acids. ClpC1 and ClpC2 proteins form Tic complex in association with integral membrane proteins, Tic110, Tic40, Tic20; intermembrane space protein Tic22; and other stromal chaperones (cpHsp70, Hsp90C) and import cytosolic protein precursors via the Toc-Tic pathway (Akita et al., 1997; Nielsen et al., 1997; Paila et al., 2015). In the stroma, aberrant imported protein precursors normally targeted toward the thylakoid membrane are degraded by ClpC (Sjogren et al., 2004). Several studies have reported the functional roles of ClpC1 and ClpC2 proteins, however, the roles of the chpaeronic part of the Clp protease composed of the two proteins in photosynthesis are still unclear. Additionally, mutant studies conducted to date have used only a single mutant of ClpC1 or ClpC2, respectively due to the lethality in the double mutant lines. In the present study, we co-suppressed *NbClpC1*/*C2* in *N. benthamiana* using one single virus-induced gene silencing (VIGS) vector and identified their roles in photosynthetic capacity using "loss-of-function" analysis.

Results

Phylogenic analysis of ClpC genes

A phylogenetic tree was drawn to elucidate the evolutionary relationships among different *ClpC* genes from the *Solanaceae* species and from *Arabidopsis* (Fig. 1A). Due to the sequence similarities in the *ClpC1* and *ClpC2* genes, *ClpC1* and *ClpC2* in *Arabidopsis* were grouped separately from *ClpC1* and *ClpC2* in *Solanaceae* species. When the VIGS sequence from *ClpC1* in *C. annuum* was compared with the *ClpC1* and *ClpC2* genes in *N. benthamiana*, it showed high sequence identities (>87%), indicating the VIGS construct based on the *CaClpC1* sequence could suppress the whole *ClpC1* and *ClpC2* genes (Fig. 1B).

Tissue-specific expression of NbClpC1/C2 in N. benthamiana

To investigate the potential expression of *NbClpC1*/*C2* in *N. benthamiana*, tissue from flowers, leaves, stems and roots were collected and total RNA was isolated, after which the levels of *NbClpC1*/*C2* mRNA were analyzed by semi-quantitative RT-PCR. Similar transcript levels were observed in flowers, leaves and stems, whereas the transcript levels were relatively low in roots (Fig. 2A).

Co-suppression of NbClpC1/C2 by pTRV2:CaClpC

We conducted functional studies of *NbClpC1*/*C2* by employing TRV-based VIGS in *N. benthamiana* (Ahn and Pai, 2008; Cho et al., 2004; Kang et al., 2010). To accomplish this, we prepared a VIGS construct using the part from *CaClpC1* gene. Semi-quantitative RT-PCR revealed that both *NbClpC1*/*C2* genes were suppressed by the single vector, pTRV2:*CaClpC* (Fig. 2B). VIGS screening revealed that co-suppression of *NbClpC1*/*C2* caused severe leaf-yellowing phenotypes with growth retardation, indicating that malformation of the chaperonic part caused abnormalities in the subcellular levels as chloroplast structure and in the individual levels as apical dominance (Fig. 2C). The *NbClpC1*/*C2* co-suppressed *N. benthamiana* developed aberrantly with comparatively more branches. The lower leaves of *NbClpC1*/*C2* co-suppressed *N. benthamiana* were broader, thicker and wider, while the upper leaves were smaller and had pleiotropic phenotypes. Throughout the vegetative growth, the leaves of *NbClpC1*/*C2* co-suppressed plants exhibited chlorotic appearance and the plants did not produce any seeds, even though they flowered.

Lowered photosynthetic rate and pigment contents by the co-suppression of NbClpC1/C2

The photosynthetic gas exchange rate and chloroplast pigments were measured to elucidate the underlying mechanism of severe chlorotic symptoms and growth retardation phenotypes of *NbClpC1*/*C2* co-suppressed *N. benthamiana*. The *NbClpC1*/*C2* co-suppressed leaves had a significantly reduced CO_2 uptake rate, while they had only a 5.1% photosynthetic rate relative to the control plants (Fig. 3A). When the contents of chlorophyll and carotenoids were measured in the *NbClpC1*/*C2* co-suppressed leaves, both pigments were significantly reduced by the co-suppression of the *NbClpC1*/*C2* genes. The sixth leaves of the *NbClpC1*/*C2* co-suppressed *N. benthamiana* contained only 6.0% chlorophyll content and 17.2% carotenoids content compared to those of the *GFP*-silenced plants (Fig. 3B and C). The maximum quantum yield (Fv/Fm) was also significantly reduced in *NbClpC1*/*C2* co-suppressed leaves, having 13.6% relative to the *GFP*-silenced leaves (Fig. 3D).

Structures of the NbClpC1/C2 co-suppressed leaves

NbClpC1/*C2* co-suppressed *N. benthamiana* had distinct morphological and physiological differences with thicker and aberrant shaped leaves. Significant differences in both stomata sizes and densities were observed for *NbClpC1*/*C2* co-suppressed *N. benthamiana* (Fig. 4A and B). *NbClpC1*/*C2* co-suppressed *N. benthamiana* contained only 16.7% of the stomata compared with *GFP*-silenced plants (Fig. 4C). When

Table 1. Primer sequences and their amplicon sizes (bp).

Gene	Primer pairs	Amplicon size (bp)
For cloning		
CaClpC1	Forward; 5'-cggaattcTTGGAGGTGGAACGTCTG*-3'	558
	Reverse; 5'-tgggctcgagCTGGCTGGAACCTCCTCT-3'	
For semi-quantitative RT-PCR		
NbClpC1	Forward; 5'-ACACCGTCTCTAAACTCATTGG-3'	347
	Reverse; 5'-CAACTCCTCAGTCACTAAGC-3'	
NbClpC2	Forward; 5'-CATCAGTGACCGTTTTCTGCCTG-3'	310
	Reverse; 5'-GTGCTGAATATCTGCTTCAGTC-3'	

* Restriction site sequences are indicated by underlined lower case letters.

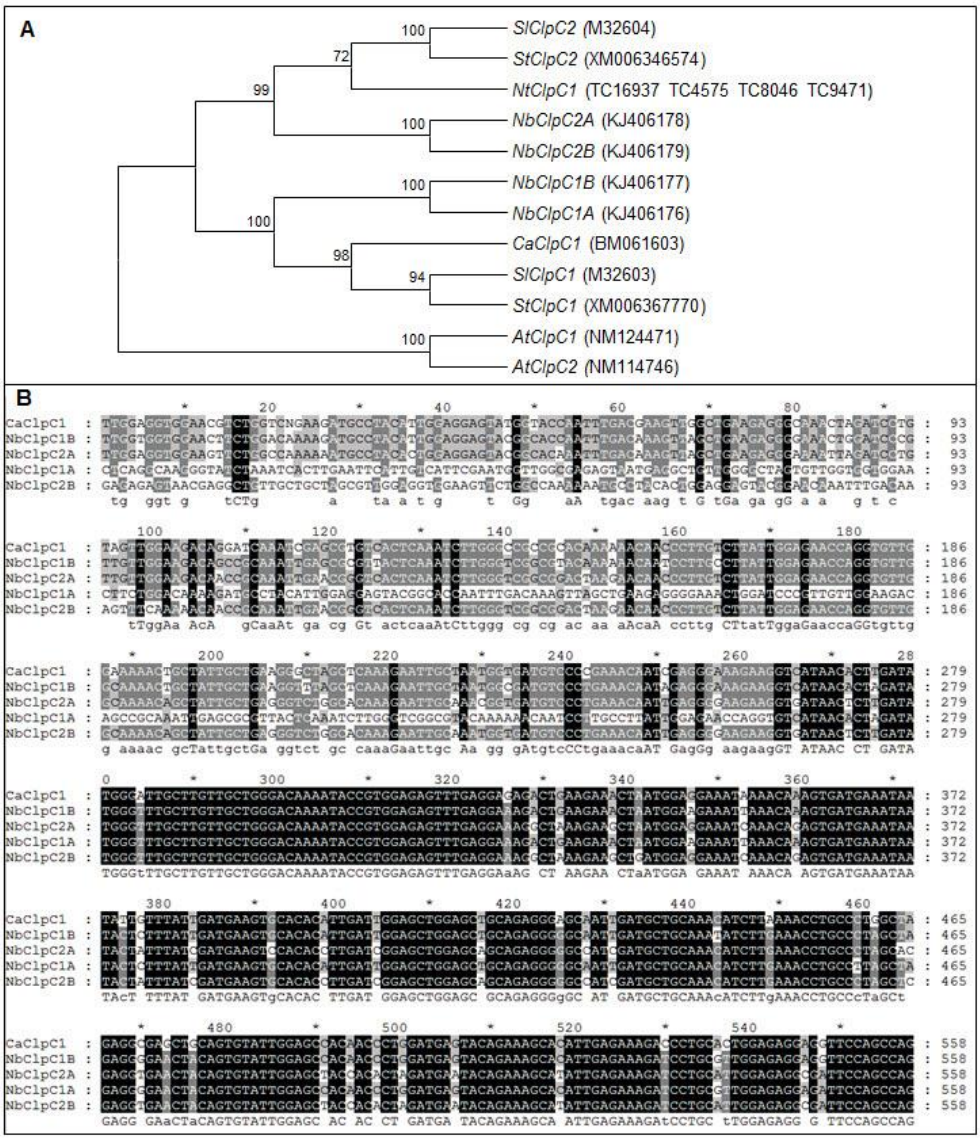

Fig 1. Phylogenetic analysis and alignment of the nucleotide sequences of *ClpC1* and *ClpC2* genes in *Solanaceae* species and *Arabidopsis*. (**A**) The phylogenic analysis for the total 12 nucleotide sequences found in *Solanaceae* species and *Arabidopsis* were done by the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The number in the bracket is the GenBank accession numbers. (**B**) The nucleotide sequence of *C. annuum ClpC1* used for the VIGS construct was aligned with *N. benthamiana ClpC1* and *ClpC2*. Identical and similar nucleotides are highlighted in black and gray, respectively.

stomatal size was calculated it revealed 2.5-fold increase of stomatal size in *NbClpC1*/*C2* co-suppressed plants relative to *GFP*-silenced plants (Fig. 4D). Owing to the distinct morphological and physiological changes in *NbClpC1*/*C2* co-suppressed *N. benthamiana*, the chlorotic symptoms and lower photosynthetic rate were further investigated by employing microscopic analysis. Microscopic observation for the leaves revealed that *NbClpC1*/*C2* co-suppressed *N. benthamiana* had coarsely packed mesophyll cells relative to the *GFP*-silenced plants (Fig. 5).

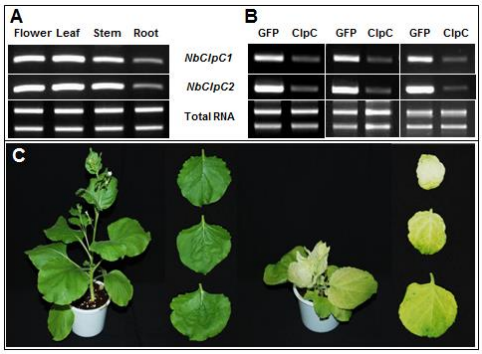

Fig 2. Expression pattern of *NbClpC1* and *NbClpC2* and gene silenced phenotype. (**A**) The expression levels for *N. benthamiana NbClpC1* and *NbClpC2* in flowers, leaves, stems and roots were measured by semi-quantitative RT-PCR. (**B**) Semi-quantitative RT-PCR analysis for the identification of silencing of *NbClpC1* and *NbClpC2* expression with three independent samples. Equivalent usage of total RNA was confirmed by rRNA staining. (**C**) Phenotypes of the *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana*. The 4th, 5th and 6th leaves of *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana* were compared from bottom to top.

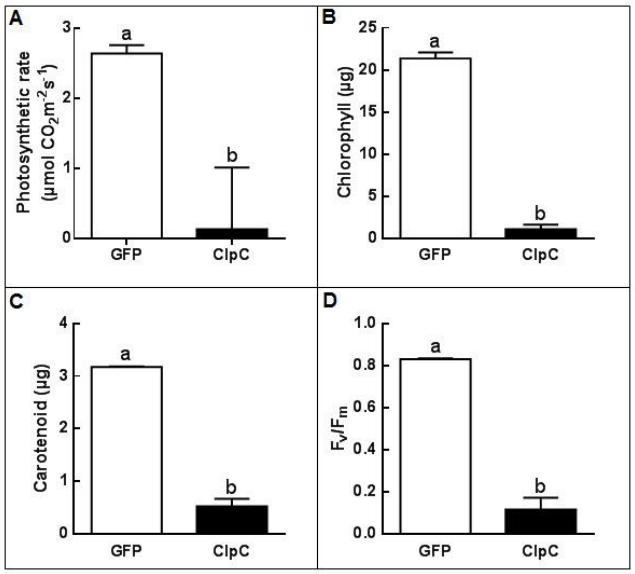

Fig 3. Photosynthetic rate, pigment contents and chlorophyll fluorescence kinetics. (**A**) Photosynthetic rate, (**B**) chlorophyll, (**C**) carotenoid and (**D**) chlorophyll fluorescence kinetics in the *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana*.

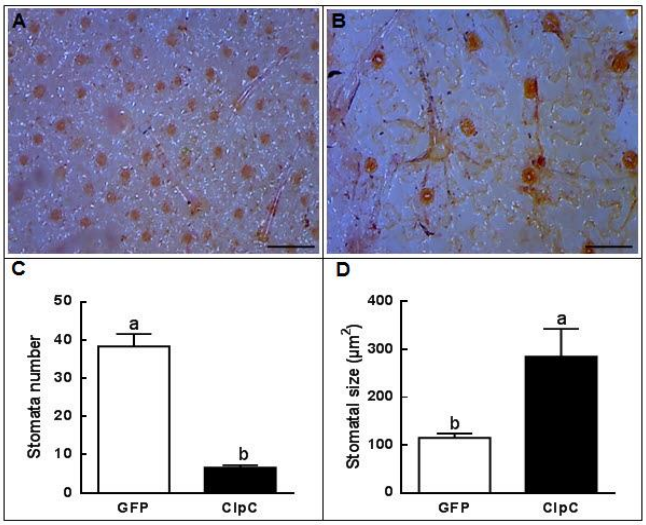

Fig 4. Stomatal numbers and sizes after co-suppression of *NbClpC1*/*C2* in *N. benthamiana*. Stomata in the abaxial surface of leaves in (**A**) *GFP*-silenced and (**B**) *NbClpC1*/*C2* co-suppressed *N. benthamiana*. (**C**) Stomatal numbers and (**D**) sizes in the *NbClpC1*/*C2* co-suppressed *N. benthamiana*. Stomata numbers were measured from a surface area of 0.25 mm^2. Bars = 50 μM.

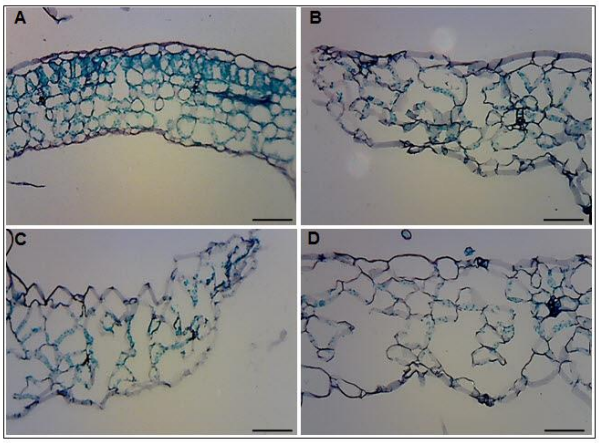

Fig 5. Cross sectional structures of the leaves. (**A**) *GFP*-silenced and (**B**-**D**) *NbClpC1*/*C2* co-suppressed *N. benthamiana*. Bars = 50 µM.

Discussion

In higher plants, there are two chloroplast-localized ClpC proteins termed as ClpC1 and ClpC2 (Sjogren et al., 2014). Bioinformatics analysis revealed that the allotetraploid *N. benthamiana* genome contains two copies of *NbClpC1* and *NbClpC2*, which share more than 93% similarity with each other at the nucleotide sequence level. The *NbClpC1* and *NbClpC2* genes also share high similarities with other *ClpC1* and *ClpC2* genes in *Solanaceae* species (Fig. 1A and B). Although *Arabidopsis* contains *clpc1* and *clpc2* mutant lines, the double homozygous genotype for *clpc1* and *clpc2* caused very early embryo lethality (Kovacheva et al., 2007). Due to the no-offspring production by the lethality in *clpc1* and *clpc2* double mutant lines, it was impossible to understand the roles of the chaperonic part in the plant development and physiology, however, the VIGS technique made it possible to develop a plant with suppressing *ClpC1* and *ClpC2* simultaneously with only one vector (Ali et al., 2015).

NbClpC1/*C2* co-suppressed *N. benthamiana* showed a distinct phenotype relative to the *GFP*-silenced plants. The pleiotropic phenotype of *NbClpC1*/*C2* co-suppressed *N. benthamiana* confirmed that *NbClpC1*/*C2* play an important role throughout the life cycle of the plants. The *NbClpC1*/*C2* co-suppressed plants showed significant growth retardation, which might be due to energy limitation caused by dramatic changes of metabolite contents in glycolysis and TCA cycle detected by our metabolome analysis (data not shown). The leaves of *NbClpC1*/*C2* co-suppressed *N. benthamiana* displayed a chlorotic appearance throughout all developmental stages. When the *GFP*-silenced plants formed seeds, *NbClpC1*/*C2* co-suppressed *N. benthamiana* began to flower. Interestingly, the flowering did not guarantee seed formation in the *NbClpC1*/*C2* co-suppressed *N. benthamiana*. Actually all of the examined seed pods did not have any seed in them (data not shown). Our results were consistent with those of Kovacheva et al. (2007), who reported that *AtClpC1* and *AtClpC2* double homozygous genotype caused very early embryo lethality. Indeed, *NbClpC1*/*C2* genes play important roles regulating the growth and development of *N. benthamiana* plants (Fig. 2C and Fig. 4B). *NbClpC1*/*C2* co-suppressed *N. benthamiana* had only a 5.1% photosynthetic rate relative to the *GFP*-silenced plants (Fig. 3A). The chloroplast division and normal development are an important determinant of the photosynthetic capacity of leaves. It has reported that co-suppression of *NbClpC1*/*C2* affected chloroplasts development, and those chloroplasts did not form a distinct inner membrane system (Ali et al., 2015). The abnormal chloroplasts may lead to impaired photosynthesis processes, and therefore decrease net photosynthesis. Indeed, impaired chloroplasts are known to have much lower photosynthetic capacity (Sjogren et al., 2004). Co-suppression of *NbClpC1*/*C2* reduced the rate of photosynthesis through abnormal chloroplast development in the early stage of development, ultimately affecting PSII activity and chlorophyll and carotenoids biosynthesis.

Co-suppression of *NbClpC1*/*C2* in *N. benthamiana* significantly affected the number of stomata, having only 16.7% of stomata relative to the control (Fig. 4C). Stomata size and density of plants changed to adjust gas exchange between the atmosphere and leaf internal air spaces based on environmental conditions. It has been reported that fewer stomata reduce transpiration and affect CO_2 and nutrient uptake, resulting in changes in metabolic pathways such as photosynthesis and respiration, as well as ion uptake, transport and extrusion (Xiong and Zhu, 2002). Reduced number of stomata resulted in reduction in the supplies of water and mineral nutrients to the entire plant system because stomata act as a gateway for efficient gas exchange and water movement from the roots through the vasculature to the atmosphere (Raven, 2002). Stomagen and EPIDERMAL PATTERING FACTOR (EPF) is a positive and negative mesophyll-to-epidermis signaling factor, which positively and negatively regulates stomata density, respectively (Sugano et al., 2010). At elevated CO_2 levels, *EPF2* was induced in the wild type and reduced stomata density, but not in *Arabidopsis thaliana* carbonic anhydrase double mutants (*ac1ca4*) (Engineer et al., 2014). CO_2 RESPONSE SECRETED PROTEASE (CRSP) also controls the number of stomata through cleavage of the EPF2 (Engineer et al., 2014). Therefore, reduced number of stomata in the *NbClpC1*/*C2* co-suppressed *N. benthamiana* indicates that ClpC is the key regulatory components for regulating the number of stomata through selective degradation of negative regulators.

The physiological states of leaves of *NbClpC1*/*C2* co-suppressed *N. benthamiana* were examined by measuring the different photosynthetic parameters that showed dramatic changes relative to those of *GFP*-silenced plants (Fig. 3D). Fv/Fm is an indication of adverse circumstances, and the reduced value of Fv/Fm indicates that a proportion of the PSII reaction center was damaged (Veres et al., 2000). The Fv/Fm is often found to be at or approaching 0.8 in healthy tissues, and tissues at this value are associated with full photosynthetic functioning (Bjorkman and Demmig, 1987; Mohammed et al., 1995; Percival, 2005). The Fv/Fm value of

NbClpC1/*C2* co-suppressed *N. benthamiana* was only 0.11, which is only 13.6% of that of *GFP*-silenced plants (Fig. 3D). Mutation of the *ClpC1* gene in *Arabidopsis* significantly reduced the PSI and PSII contents (Sjogren et al., 2004). Since *Arabidopsis* with malfunctioning ClpC1 subunits did not undergo proper formation of active Clp protease (Sjogren et al., 2004), abnormally developed chloroplasts due to co-suppression of *NbClpC1*/*C2* genes should not be able to degrade misfolded/damaged proteins, eventually hampering proper development of the PSII reaction center. However, it is not clear whether Clp protease is involved in the degradation of PSII in *Chlamydomonous reinhardtii* (Majeran et al., 2001). The specific decrease in the Fv/Fm value of *NbClpC1*/*C2* co-suppressed *N. benthamiana* suggests that ClpC1 and ClpC2 could participate in the biogenesis and turnover of PSI and PSII in *N. benthamiana* chloroplasts. Co-suppression of *NbClpC1*/*C2* in *N. benthamiana* significantly reduced the chlorophyll and carotenoids contents (Fig. 3B and C). In plastids, geranylgeranyl pyrophosphate (GGPP) is the common substrate for biosynthesis of chlorophyll (Wettstein et al., 1995), carotenoids (Bartley and Scolnik, 1995) and gibberellin (Andrew, 1998). Chlorophyll biosynthesis is tightly coupled with thylakoid membrane biogenesis and accumulation of chlorophyll-protein complexes (Hartel et al., 1997; Reinbothe and Reinbothe, 1996). When chlorophylls were absent from *Chlamydomonas*, the light-harvesting chlorophyll binding (LHCB) proteins were absent (Eggink et al., 2001). Chlorophyll was required to import LHCB proteins into chloroplasts because their importation was impaired without these pigments (Hoober et al., 2007). Only a small number of LHCB proteins were found within chloroplasts when the rate of chlorophyll biosynthesis was low (Hoober et al., 2007). Microscopic observation revealed that *NbClpC1*/*C2* co-suppressed *N. benthamiana* had coarsely packed mesophyll cells (Fig. 5B-D) that contained malfunctioning chloroplasts (Ali et al., 2015). Since the synthesis of chlorophylls and carotenoids occurs in chloroplasts, malfunctioning chloroplasts could lead to a reduction in chlorophyll. Accordingly, it is possible that biogenesis was impaired due to a significant reduction of chlorophyll in the thylakoid membrane (Hartel et al., 1997; Reinbothe and Reinbothe, 1996). All evidences including the dwarf phenotype, leaf chlorosis and lower photosynthetic rates of *NbClpC1*/*C2* co-suppressed *N. benthamiana* are consistent with abnormal chloroplasts development. Construction of the thylakoid membranes and incorporation of photosynthetic complexes are vital to the developing chloroplasts, and these processes are dependent on the coordinated expression of genes encoded in both nuclear and plastid genomes, which are governed by complex signaling networks (Pogson and Albrecht, 2011). The protein import machinery in chloroplasts plays key roles in the biogenesis of chloroplasts via mediation of the import and assembly of thousands of nuclear-encoded proteins into the chloroplasts. Microscopic observation revealed that *N. benthamiana* co-suppressed *NbClpC1*/*C2* contained loosely packed mesophyll cells with less numbers of chloroplasts (Fig. 5B-D). The chloroplasts did not form distinct inner membrane systems, indicating that simultaneous suppression of the *ClpC1*/*C2* genes leads to suppression of the proper division of chloroplasts in the early stage of development (Ali et al., 2015). As shown in the evidences that ClpC1 and ClpC2 proteins play important roles in the protein import process (Akita et al., 1997; Nielsen et al., 1997; Paila et al., 2015), co-suppression of both genes can hinder more seriously the import process, assembly of the imported proteins, degradation of unwanted and/or damaged proteins, therefore, can exert the effect of suppression as abnormal development of leaves with less numbers of chloroplasts.

Materials and Methods

Plant growth conditions

N. benthamiana seeds were sown and grown in a mixture of coco peat (70%), peat moss (17%), zeolite (5%) and perlite (8%) contained in plastic pots (12 cm diameter x 10 cm height). Plants were regularly watered and grown under fluorescent lights at 120 µmol of photons $m^{-2}s^{-1}$ under a regime of 16/8 h light/dark at 22±2°C in an environmentally controlled walk-in chamber.

VIGS of NbClpC1/C2 in N. benthamiana

For the co-suppression of *NbClpC1*/*C2* genes in *N. benthamiana*, a VIGS vector was constructed by insertion of a 558 bp of *CaClpC1* gene from pepper (*Capsicum annuum* cv. Bukang) into the multi-cloning site of the pTRV2 vector. The 558 bp fragment was generated by PCR using the forward primer and the reverse primer for *CaClpC1* cloning (Table 1). The PCR fragment was digested with *EcoR1* and *Xho1* restriction enzymes and then cloned into the pTRV2 vector to generate the pTRV2:*CaClpC* plasmid. The pTRV1, pTRV2:*CaClpC* and pTRV2:green fluorescent protein (pTRV2:*GFP*) were transformed into *Agrobacterium tumefaciens* strain GV2260 by the freeze-thaw method (Chung et al., 2004; Liu et al., 2002). Overnight cell cultures of *A. tumefaciens* transformed with pTRV2:*CaClpC*, pTRV2:*GFP* and pTRV1 were centrifuged at 3,000 rpm for 10 min, after which the pellets were resuspended in 10 mM MES and $MgCl_2$ to give an absorbance of 0.5 at OD_{600} for pTRV2 constructs and 0.1 for pTRV1. The *A. tumefaciens* with pTRV1 and pTRV2 was added to acetosyringone at a final concentration of 100 µM, after which the samples were incubated at 22°C for 4 h, then mixed at a 1:1 ratio. The mixed *A. tumefaciens* was subsequently infiltrated into the lower two leaves of four leaf stage (18 days old) *N. benthamiana* using the barrel of a 1 mL needleless syringe. The treated plants were then grown at 22±2°C under a 16/8 h light/dark cycle. All experiments were performed using the 6^{th} leaves above the infiltrated leaves from the *GFP*-silenced or *NbClpC1*/*C2* co-suppressed *N. benthamiana* after 4 weeks of infiltration.

Phylogenic analysis of NbClpC1/C2 gene

The phylogenic relation of *ClpC1* and *ClpC2* genes from the *Solanaceae* species and *Arabidopsis* was analyzed by employing the MEGA software version 6.0 method (Blair et al., 2008; Tamura et al., 2013; Winnepenninckx et al., 1995). Bootstrap analyses were performed on each dataset using neighbor-joining with 1,000 replicates. Multiple alignment of the nucleotide sequences of *C. annuum ClpC1* homolog with *NbClpC1*/*C2* was carried out using the ClustalX and the Genedoc sequence alignment program. GenBank accession numbers for the sequences were are as follows: *CaClpC1* (*C. annuum ClpC1*, BM061603), *NbClpC1A* (*N. benthamiana ClpC1A*, KJ406176), *NbClpC1B* (*N. benthamiana ClpC1B*, KJ406177), *NbClpC2A* (*N. benthamiana ClpC2A*, KJ406178), *NbClpC2B* (*N. benthamiana ClpC2B*, KJ406179), *SlClpC1* (*Solanum lycopersicum ClpC1*, M32603), *SlClpC2* (*S. lycopersicum ClpC2*, M32604), *StClpC1* (*S. tuberosum ClpC1*, XM006367770), *StClpC2* (*S. tuberosum ClpC2*,

XM006346574), *AtClpC1* (*A. thaliana ClpC1*, NM124471) and *AtClpC2* (*A. thaliana ClpC2*, NM114746). *NtClpC1 (N. tabacum ClpC1*) was prepared by aligning the sequences of TC16937, TC4575, TC8046 and TC9471 from the Gene Index Project, Harvard University.

Transcript analysis

Differential expression of *NbClpC1*/*C2* was measured by semi-quantitative RT-PCR. One µg of total RNA isolated from the sixth leaves above the infiltrated leaves using Trizol™ reagent (Molecular Research Center, Cincinnati, OH, USA) was used to synthesize 20 µL of cDNA by the GoScript™ reverse transcription system (Promega, Madison, WI, USA). Semi-quantitative RT-PCR was performed using 0.5 µL of the newly synthesized cDNA with gene-specific primers (Table 1). The cDNA was mixed with HiPi PCR premix (Elpis Biotech, Korea), after which PCR was conducted by subjecting the samples to pre-denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 10 sec, annealing at 50°C for 40 sec and extension at 72°C for 40 sec, and then final extension at 72°C for 5 min using a PCR machine (XP Thermal Cycler, BIOER, China). The amplified PCR products were visualized on 1.2% agarose gel.

Leaf gas exchange rate

To investigate the photosynthetic competence of *NbClpC1*/*C2* co-suppressed leaves, the photosynthetic CO_2 exchange rate of both *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana* was measured on the middle parts of the 6th leaves using the LI-6400XT portable photosynthesis system (LI-COR, Nebraska, USA).

Total leaf chlorophylls and carotenoids content

The 6th leaves from *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana* were ground with a mortar and pestle in 2 mL cold acetone/Tris buffer solution (80:20 v/v, pH = 7.8), then centrifuged to remove particulates, after which the supernatant was diluted to a final volume of 6 mL by the addition of acetone/Tris buffer. The absorbance of the extract solutions was measured using an UV/VIS spectrophotometer (Optizen 2120UV, Mecasys Co., Ltd., Korea). Total chlorophyll and total carotenoids concentrations were determined according to the equation described by Lichtenthaler and Wellburn (1983).

Chlorophyll fluorescence yields

Leaf discs from the 6th leaves of *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana* were dark-treated for 30 min in the leaf clips of a plant efficiency analyzer (Hansatech, King's Lynn, UK) and the excitation light was administered at 80% of the maximum (red light, peak at 650 nm, 2800 µmol of photons $m^{-2}s^{-1}$) for 2 s using the plant efficiency analyzer (Choi et al., 2001). The maximum efficiency of PSII was estimated by a chlorophyll fluorescence ratio, Fv/Fm, where Fv is variable fluorescence after dark incubation and Fm is maximum fluorescence (Choi et al., 2001).

Microscopic observation of stomata and leaves

The abaxial epidermis of the middle part of mature leaves were cleaned first, peeled off from the leaf surface and stained with Safranin-O. Images were taken using a light microscope (EX30, Ningbo Sunny Instruments, China). Stomata numbers were counted on three randomly selected areas of *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana* and stomatal densities were calculated. Stomatal size was measured using the photomicrograph and then calculated as guard cell length multiplied by the width of the guard cell pair (Franks and Beerling, 2009). Leaf stomatal density was expressed as the number of stomata per unit leaf area (Radoglou and Jarvis, 1990).

To study the leaf structures of *GFP*-silenced and *NbClpC1*/*C2* co-suppressed *N. benthamiana*, segments of a leaf (1×3 mm^2) were immersed in formaldehyde-acetic acid-ethanol fixative (50% ethanol, 5% glacial acetic acid, 10% with 37% formaldehyde and 35% ddH_2O) at 4°C for 10 h. Samples were then dehydrated using ethanol and processed in ethanol-xylene solutions for 30 min each. The samples were subsequently incubated in 100% xylene for 30 min, after which they were infiltrated in paraffin wax, arranged in a hot boat and molded with paraplast. Sections (5-8 µM) of the paraffin block were made using a rotary microtome (HM 355 S, Microm, Heidelberg, Germany). The paraplasts were then removed from the ribbons by dipping the slides with ribbons into 100% xylene for 30 min, followed by 50% xylene: 50% ethanol for 10 min, and 100% ethanol for 5 min, after which the samples were rehydrated in an ethanol series and ddH_2O. Next, samples were stained with 0.5% Heidenhain hematoxylin, washed with ethanol-xylene solutions and mounted with glycerol. The mounted samples were then observed with a light microscope.

Statistical analysis

All experiments were performed in at least three replicates and conducted three times. The data are presented as the mean ± standard deviation of three replicates. The variance of sample data was analyzed by ANOVA using the Statistical Analysis Software (SAS) version 9.2 (SAS Inc., Cary, NC, USA).

Conclusions

Our data demonstrate that ClpC1 and ClpC2 play important roles in photosynthesis via abnormal development of leaves with less numbers of stomata and chloroplasts. The severe chlorosis, aberrant structure and semi-dwarf phenotype observed induced by the co-suppression of ClpC1 and ClpC2 may have occurred due to less content of chlorophylls and carotenoids. The reduction of photosynthetic capacity in the *NbClpC1*/*C2* co-suppressed *N. benthamiana* supports the involvement of Clp protease in the biogenesis and development of functional chloroplasts.

Authors' contributions

Experimental works were done by SA, YY, YC and RD. Experimental design and data interpretation were done by WO, KB, YP and JC. Manuscript was prepared by SA, YY and KB.

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